

# A Simple and Rapid Technique for Identification of Large Numbers of Individual Mosquitoes Using DNA Hybridization

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A general method for obtaining species-specific repetitive DNA sequences is described. The method is based on the detection of recombinant DNA clones containing repetitive sequences using labeled total genomic DNA. These repetitive DNA sequences can be used to identify individual mosquito adults, pupae, and larvae squashed on filter membranes (squash blots). This technique was used to distinguish individuals of the four sibling species of the *Anopheles quadrimaculatus* complex. Repetitive DNA sequences and squash blots can be of use for rapid identification of other insect species in field collections.

**Key words:** sibling species, DNA probes, *Anopheles quadrimaculatus*

## INTRODUCTION

Fast and accurate identification of insects in field collections is essential to ecological research and for making practical decisions regarding insect control. Two important examples are African/European honeybees, which are so closely related that reliable morphological identification requires computer assistance [1], and the mosquitoes of the *Anopheles gambiae* species complex, which are usually distinguished by the technique of polytene chromosome analysis [2]. A rapid, simple, and easily interpretable screening system that can distinguish closely related insect species will speed research on wild populations and improve insect control decisions.

In this paper I describe techniques for the isolation of species-specific repetitive DNA sequences and the use of these sequences to identify individual

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insects squashed on filter paper. Nitrocellulose filters on which individual insects have been squashed bind sufficient DNA to be detected using standard hybridization techniques. This procedure is used in conjunction with species-specific repetitive sequences to develop a method for the simple and rapid identification of individual mosquitoes of the *Anopheles quadrimaculatus* complex. It could potentially be used to identify individual insects of any species.

## MATERIALS AND METHODS

### Source of Mosquitoes

Identification of *A. quadrimaculatus* species A, B, C, and D mosquitoes from mixed populations was by polytene chromosome patterns or by isozyme analysis (performed by P. Kaiser or S. Narang). Species A mosquitoes [3] were primarily laboratory-reared ORLANDO colony strain; with additional specimens as indicated from Lake Panasofkee, Florida, a population consisting of more than 99% species A; from near College Station, Texas, a population consisting essentially entirely of species A; and from New York state, consisting essentially entirely of species A. Species B mosquitoes [3] were adults reared in the laboratory from eggs laid by gravid female mosquitoes collected from Octahatchee, Florida. Species C mosquitoes [4] were from Bear Bay Swamp, Florida, a population consisting of 99% species C. Species D mosquitoes [5] were adults reared in the laboratory from eggs laid by gravid female mosquitoes collected from north Florida.

### DNA Isolation

Isolation of mosquito DNA was previously described [6]. Briefly, 1 g of mosquitoes was frozen at  $-80^{\circ}\text{C}$  and powdered with a mortar and pestle. The powder was rapidly dispersed in 100 ml ice-cold 0.1 N NaCl, 0.2 M sucrose, 10 mM EDTA, 30 mM Tris-HCl, pH 8.0, and lightly ground using a few strokes in a glass homogenizer. To lyse the cells, 25 ml 0.25 M EDTA, 2.5% SDS,\* 0.5 M Tris-HCl, pH 9.2 was blown into the homogenate using a syringe and about 1 mg of proteinase K added. The mixture was incubated at  $55^{\circ}\text{C}$  for 1 h. To precipitate SDS and undigested protein, 17 ml of 8 N potassium acetate was added. Following incubation on ice for 1 h, the mixture was centrifuged at 20,000g for 10 min. DNA was precipitated from the supernatant by addition of two volumes of ethanol and centrifugation at 20,000g for 10 min. The precipitate was washed with 70% ethanol, dried, and redissolved in 10 ml 1% sodium lauryl sarcosinate, 1 mM EDTA, 10 mM Tris-HCl, pH 8.0. DNA was further purified by CsCl centrifugation and phenol extraction [7].

### Library Construction

The construction of mosquito genomic DNA libraries and the screening techniques used for the isolation of Arp2, Brp1, and Crp1 were previously described [8]. *Sau*3A partial digest fragments were purified by centrifugation through 5% to 24% NaCl gradients. Fragments of the appropriate size (10–20 kb) were collected and ligated to *Bam*HI cut  $\lambda$  EMBL 3A arms (Stratagene, La Jolla, CA) according to manufacturer's recommendations. Aliquots of the ligated DNA

\*Abbreviations used: SDS = sodium docetyl sulfate;  $1 \times$  SSPE = 0.15 N NaCl, 0.01 N  $\text{NaH}_2\text{PO}_4$ , 0.001 M EDTA; kb = thousand base pairs.

were packaged using GigaPack Gold (Stratagene, La Jolla, CA) and plated on *Escherichia coli* strain P2 392 according to manufacturer's recommendations.

### Library Screening

Filter lifts were conducted according to standard procedures [7]. Recombinant phage were plated at  $10^2$  to  $10^3$  plaques/plate. After lysis, plates were refrigerated and nitrocellulose filter lifts conducted according to standard methods. Filters were denatured in 0.5 N NaOH, 1.5 N NaCl, and neutralized in 1.5 N NaCl, 1 M Tris-HCl, pH 8.0. Filters were baked at 80°C under vacuum and hybridized as below. Total genomic DNA was labeled with  $^{32}\text{P}$  by nick translation using a kit (BRL, Gaithersburg, MD).

### Squash Blots

The squash blot procedure was based on a published procedure [9]. Mosquitoes were arranged in a grid pattern on damp nitrocellulose membrane (BA 85, Schleicher and Schuell, Keene, NH). The mosquitoes were covered with a second nitrocellulose membrane and thoroughly squashed by rolling a metal rod over the filters. The resulting sandwiches were laid on 3-MM filter paper (Whatman) saturated with denaturing solution (0.5 N NaOH, 1.5 N NaCl) for approximately 5 min per side. Then they were transferred onto 3-MM paper saturated with neutralizing solution (1 M Tris-HCl, pH 8.0, 1.5 N NaCl) for 5 min per side. The two membrane filters were separated and air dried (with the mosquito side up). The filters were baked at 80°C under vacuum to fix DNA to the filter. During prehybridization, the filters were gently brushed to remove the bulk of the adhering mosquito bodies.

### Hybridization

For prehybridization, filters were immersed in blocking solution (2% nonfat dry milk, 0.2% SDS) at 55°C for several hours. Treated filters were hybridized to denatured nick-translated probes. Hybridization conditions were 30% formamide,  $5\times$  SSPE, 1% SDS, 1% nonfat dry milk at 55°C overnight. After hybridization, filters were thoroughly washed in  $1\times$  SSPE, 0.2% SDS at 55°C and air dried. Hybridization was detected by autoradiography. Following autoradiography, probe DNA was removed by briefly immersing the filters in boiling water and the squash blots reprobed with alternate sequences.

## RESULTS

### Isolation of Differentially Hybridizing Repetitive DNA Sequences

Cockburn and Mitchell [8] showed that cloned repetitive insect DNA sequences can be detected by hybridization to labeled total genomic DNA from the species from which the library was constructed. Under the conditions used, only clones containing sequences represented  $> 30$  times/genome hybridized detectable amounts of genomic DNA. Some repetitive sequences differed even between closely related species, and most repetitive sequences differed between genera. These observations suggested the following simple method for screening for species-specific repetitive sequences.

A recombinant DNA library is constructed for the insect in question. In gen-

eral, the length of the inserts should be less than the distance between two repetitive sequences in the genome so that each clone has at most one repetitive sequence. Insert lengths of 10 kb or more would be suitable for insects that have widely spaced repetitive sequences, such as anopheline mosquitoes [8]. For insects that have closely spaced repetitive sequences, such as *Aedes* mosquitoes [8], it will be necessary to use much shorter insert lengths, possibly < 1 kb. Duplicate subsets of the library are screened with the genomic DNA used to construct the library and with genomic DNA from closely related species. Since the sequences being sought are repetitive, they will be present many times in the library and it is not necessary to screen the entire library. Clones detectably hybridizing only to the genomic DNA used to construct the library are isolated. These can be further characterized to determine if they are suitable for use as species-specific probes.

This protocol was tested using the *A. quadrimaculatus* complex, which consists of four morphologically indistinguishable sibling species (species A, B, C, and D). These species, which have been extensively studied by Seawright and co-workers [3–5], can be distinguished based on isozyme electrophoresis [3–5], chromosome polymorphisms [4], hybrid sterility [3], and mitochondrial DNA restriction fragment polymorphisms (S. E. Mitchell and A. F. C., unpublished information). Recombinant DNA libraries of *A. quadrimaculatus* species A, B, and C [8] were screened with total DNA of each of the four species of the complex (insufficient species D individuals were available for library construction). Most of the repetitive DNA sequences hybridized to all of the four genomes. In each case, however, at least one sequence was found that hybridized primarily to the DNA used to construct the library. An example of such a differential screening is shown in Figure 1. One representative differ-

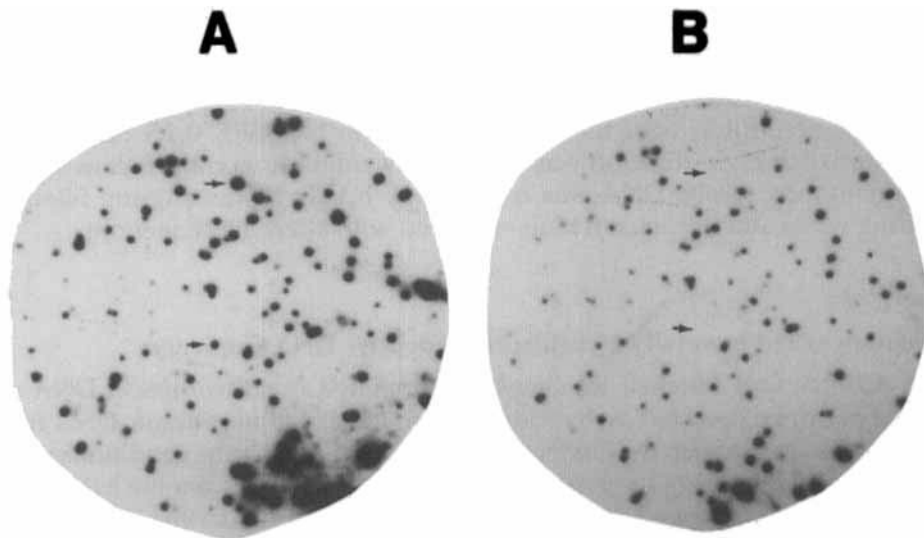


Fig. 1. Hybridization of total mosquito DNA to plaque lifts from a species A genomic library. Probes: (A) species A; (B) species B. Arrows indicate differentially hybridizing plaques. Arp2 was isolated in this screen.

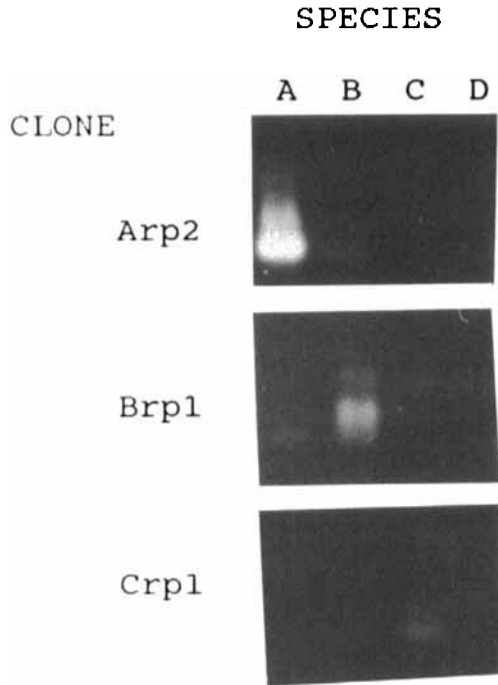
HYBRIDIZATION OF SPECIES-SPECIFIC  
CLONES TO MOSQUITO DNA

Fig. 2. Probing of *A. quadrimaculatus* species complex with species-specific probes. DNA extracted from several hundred individuals of each species was electrophoresed on duplicate agarose gels and probed with the three species specific probes. (A) Species A (ORL), (B) species B (lab reared families), (C) species C (Bear Bay Swamp, Florida), (D) species D (lab reared families).

entially hybridizing clone from each library was isolated and further characterized. These are Arp2, Brp1, and Crp1.

**Species Specificity of Differentially Hybridizing Repetitive Sequences**

The cloned species-specific DNAs were used to probe filters containing purified DNA isolated from species A, B, C, and D (Fig. 2). Arp2 hybridized intensely with species A DNA but also slightly with species B DNA. No hybridization is seen with either species C or species D DNA. Brp1 hybridizes to species B DNA but also slightly to species A DNA. No hybridization is seen with either species C or species D DNA. Crp1 hybridizes only to species C DNA, and no hybridization is seen to any of the other DNAs. Therefore, despite the slight hybridization of Arp2 to species B and Brp1 to species A, the three probes can be used as follows to differentiate the DNAs of the four species: (1) species A hybridizes more intensely to Arp2 than Brp1 and not to Crp1, (2) species B hybridizes more intensely to Brp1 than Arp2 and not to Crp1, (3) species C hybridizes only to Crp1, and (4) species D does not hybridize to any of the three probes.

### Squash Blots

The DNA preparations used in the construction of the libraries and the isolation of the species-specific probes were isolated en masse from hundreds of individual mosquitoes. These sequences could have been present at a very high copy number in a few individuals of the appropriate species and absent in others. Alternatively, they could have been present in approximately equal numbers in all individuals of that species. It is also possible that a few individuals of the other species had copies of the sequences, but that these were diluted by individuals that lacked the sequences. To examine these questions, it was necessary to look at the genomes of individual mosquitoes.

Arp2, Brp1, and Crp1 were used to probe squash blots of *A. quadrimaculatus* species A, B, C, and D. Some probe preparations gave high backgrounds or weak hybridization, which could be due to the small proportion of species-specific sequences in the clones. This problem should be eliminated by subcloning of the species-specific sequence into plasmid vectors. A sample of the results is shown in Figure 3 and the complete data are summarized in Table 1. The hybridization with individual insects is essentially identical to the results with pooled samples. These sequences are distributed in approximately equal amounts in most or all individuals of the species in which they occur, not in varying amounts. Therefore they should be useful for identifying individual mosquitoes.

When a large number of individuals of the same species was being screened at a high density on the filter, it was difficult to determine whether all of the individuals hybridized to the appropriate probe. Individuals were scored as positive only when it was clear that they hybridized, so the negative results with homologous probes reported in Table 1 are probably an overestimate of the actual number of anomalous negatives. In contrast, it was easy to detect

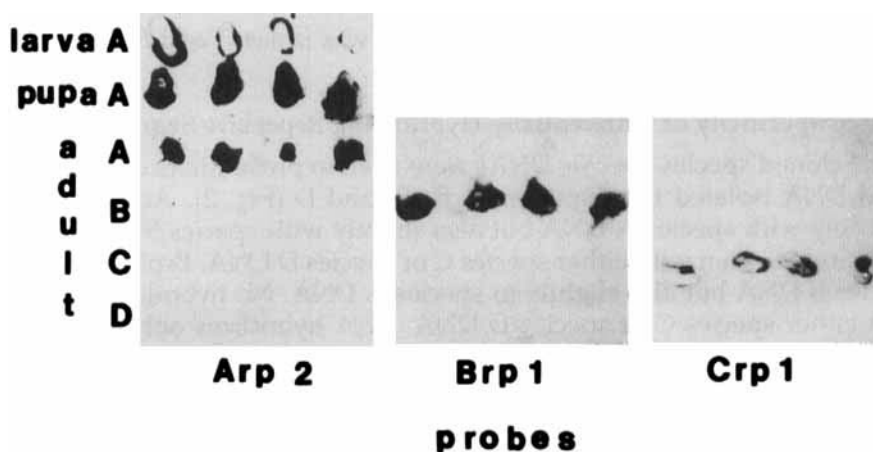


Fig. 3. Hybridization of Arp2, Brp1, and Crp1 to squash blots. Each strip has four individuals of each species (species A = ORL strain). Arp2 squash blot also has four larvae and four pupae of species A ORL strain.

TABLE 1. Hybridization of Species-Specific Probes to Squash Blots\*

Species	Probe											
	Arp				Brp				Crp			
	+++	++	+	-	+++	++	+	-	+++	++	+	-
A	271	0	5	0	0	0	170	81	0	0	2	83
B	0	0	5	129	99	30	5	0	0	0	47	87
C	0	1	0	127	0	0	0	103	124	0	4	0
D	0	0	2	53	0	0	0	55	0	0	0	55

\*The results from several experiments are summarized. The results for the different populations and life stages of species A did not differ significantly and are pooled. The results for the dried mosquitoes were omitted, as they were less intense in all cases.

single positive hybridizations in a group of negative hybridizations. Only one anomalous positive result was seen, an individual (perhaps a species A/C hybrid) that hybridized strongly to both Arp2 and Crp1. This individual was scored as species C, which accounts for the single positive hybridization of the Arp2 probe to species C.

#### Geographic Distribution of Arp2 Sequences in Species A

The library construction, screening, and initial characterization of Arp2 were performed using the ORLANDO colony strain, which has been isolated from the wild for decades. To test the possibility that Arp2 was fixed during colonization, several populations of species A mosquitoes from the extreme edges of its range were tested. Individuals from Florida, Texas, and New York all hybridized similarly to the ORLANDO colony strain. There appears to be little or no geographical diversity in the distribution of the Arp2 sequence. These same filters were also hybridized to Brp1 and Crp1 to determine if those sequences were present in wild populations of species A. The hybridizations were negative, as with the ORLANDO colony strain.

#### Squash Blots with Larva and Pupa

Because DNA sequences do not change during development, these same sequences are also present in larva and pupa. Therefore the squash blot technique was tried with fourth instar larvae and with pupae. The results were comparable to those from adults, although larvae gave smaller squashes and consequently less hybridization (Fig. 3). Specific hybridization was easy to detect, so different life stages can be mixed on the same squash blot and analyzed together.

#### Preservation of Specimens

The mosquitoes used in the preceding experiments were preserved at  $-80^{\circ}\text{C}$  until just before squashing. It is not usually practical to preserve specimens in this way for analysis—it would be especially useful to be able to analyze specimens from light traps, which are damaged and often have been dead for a day or more. I tested adult mosquitoes taken from the bottom of a mosquito population cage and which had been dead for several days. These were squashed on squash blots and analyzed. Because they had dried out, not as much material was squashed onto the membrane. The signals were less intense, but could still be interpreted.

## DISCUSSION

We have identified and cloned in bacteria DNA sequences that are present many times in the genome of *A. quadrimaculatus* species A, B, or C but are not present or are rare in the genomes of the other species. These sequences can be used as probes against DNA of individual mosquitoes and the presence/absence of hybridization used for species identification. A similar procedure was used to identify *Drosophila melanogaster* strains containing the P element transposon [10]. Since no complicated sample processing is involved, thousands of insects can be quickly and easily screened. The possibility thus exists that squash blots can be used to identify the species of individual insects and at the same time to determine if the insects are infected with particular pathogens (our technique produces two identical squash blots from each group of insects). This information would be of great utility in determining the contributions of the different members of a species group to spreading disease, and would also be important in making practical decisions about when and where to try to control the insect population.

The use of DNA probes and squash blots is much more labor efficient for screening large numbers of individual insects than the other techniques that have been used to identify the species of the *A. quadrimaculatus* complex. It is possible for a single person to screen thousands of individuals per day, since each filter can carry hundreds of individuals.

The inserts in the clones described are all > 10 kb in length. Parts of these inserts have subcloned and the repetitive portions are sequenced. The use of shorter, more defined sequences may reduce the nonspecific background seen with both the Arp2 and Brp1 probes. In addition, sequencing will allow the use of synthetic oligonucleotide probes, eliminating the need for propagation of clones.

Large-scale screening of squash blots led to the observation that it is extremely easy to detect a few positively hybridizing individuals in an otherwise negative population, but very difficult to detect a negative individual in an otherwise positive population. It is also very difficult to confirm weakly positive individuals in an otherwise strongly positive population when screening with a high density of insects on the filter. (We are currently developing an improved squashing method that gives more discrimination with high densities of mosquitoes.) Even when screening at such high density that the squashed mosquitoes overlap, a rare positive will give a strong signal. The capacity to easily detect rare individuals would be especially useful when monitoring for introduction of exotic pests.

While isolation and characterization of the species-specific probes require a well-equipped molecular biology facility, squashing and detection of mosquitoes are relatively simple, inexpensive, and easy to interpret. Unlike other biochemical identification techniques, squash blots using species-specific probes give identical results using all life stages. In addition, the state of preservation of the samples is not critical, and even insects that have been dead and at room temperature for several days can be used.

We have used standard DNA detection methodology, which involves radio-labeling the probe and detection by autoradiography. Other methods of DNA labeling are available, including fluorescent, chemical, and enzyme linkage.



Any of these methods would probably be more useful for a field assay than radiolabeling. We are currently testing fluorescent probes, which would permit the use of several differently labeled probes in the same hybridization, eliminating the need for reprobing of filters. Fluorescent labeled probes would also facilitate automated identification, abolishing the need for expert interpretation.

## LITERATURE CITED

1. Daly HV, Hoelmer K, Norman P, Allen T: Computer-assisted measurement and identification of honey bees (Hymenoptera: Apidae). *Ann Ent Soc Am* 75, 591 (1982).
2. Coluzzi M: Chromosomi politenici delle cellule nutrici ovariche nel complesso gambiae del genere *Anopheles*. *Parasitologia* 10, 179 (1968).
3. Lanzaro GC: Use of enzyme polymorphism and hybridization crosses to identify sibling species of the mosquito, *Anopheles quadrimaculatus* Say. PhD dissertation, University of Florida, p 92 (1986).
4. Kaiser PE, Smittle BJ, Seawright JA, Kline D: A new member of the *Anopheles quadrimaculatus* complex, species C. *J Am Mosq Control Assoc* 4, 494 (1987).
5. Narang SK, Kaiser PE, Seawright JA: Identification of species D, a new member of the *Anopheles quadrimaculatus* complex: A biochemical key. *J Am Mosq Control Assoc* 5, 317 (1989).
6. Cockburn AF, Seawright JA: Techniques for mitochondrial and ribosomal DNA analysis of anopheline mosquitoes. *J Am Mosq Control Assoc* 3, 261 (1988).
7. Maniatis T, Fritsch EF, Sambrook J: *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Press, Cold Spring Harbor, NY, p 545 (1982).
8. Cockburn AF, Mitchell SE: Repetitive DNA interspersal patterns in Diptera. *Arch Insect Biochem Physiol* 10, 105 (1989).
9. Kirkpatrick BC, Stenger DC, Morris TJ, Purcell AH: Cloning and detection of DNA from a nonculturable plant pathogenic mycoplasma-like organism. *Science* 238, 197 (1987).
10. Tchen P, Anxolabehere D, Nouaud D, Periquet G: Hybridization to squashed flies: A method to detect gene sequences in individual *Drosophila*. *Anal Biochem* 150, 415 (1985).